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(54) Title: HERPES-SYMPLEX-VIRUS TYPE 2 ICP4 PROTEIN AND ITS USE IN A VACCINE COMPOSITION

(57) Abstract

Immediate early HSV-2 viral protein ICP4 recognised by cytolytic T-lymphocyte (CTL) cells in humans, methods for preparation thereof and use in vaccine.

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HERPES-SYMPLEX-VIRUS TYPE 2 ICP4 PROTEIN AND ITS USE IN A VACCINE COMPOSITION

The present invention relates to therapeutic and prophylactic vaccines, novel antigens for use in such vaccine(s), methods for their preparation and their use in human medicine. In particular the present invention relates to antigens from Herpes Simplex (HSV) capable of stimulating a cytotoxic T lymphocyte response.

HSV causes lifelong infection and recurrent disease in man. There are two closely related serotypes of HSV, these are known as HSV-1 and HSV-2 respectively. In primary infections, after replication at a skin or mucosal site, the virus moves to the dorsal root ganglia and usually enters a latent phase. Reactivations then occur after appropriate stimuli, resulting in vesicles and ulcers at the mucocutaneous sites innervated by the ganglia. While neutralizing antibodies are shown to protect against primary infection and disease, their presence has no effect on the course or frequency of recurrent herpetic disease. T cell mediated immune responses, particularly of the delayed type hypersensitivity (DTH) or cytolytic (CTL) effector types have also been shown to protect against primary disease in mouse animal models. Furthermore, individuals with compromised T cell functions may undergo severe and sometimes life-threatening herpetic disease. These observations suggest a central role for effector T cell functions in control of herpes virus infections in man.

The major surface glycoproteins of Herpes Simplex Virus, gD and gC have been suggested for use in vaccines (EP 139 417 Genentech). These primarily stimulate a neutralising antibody response.

Since the mechanism of antigen recognition by CTL involves breakdown of native antigen into peptides, binding of the proteolytic fragments to MHC molecules and export of the complex to the cell surface, any virus coded polypeptide, not just those that are integral membrane proteins like the glycoproteins, can be a potential target of T cell mediated responses. However since the HSV genome codes for several non structural proteins and internal virion proteins, in addition to external glycoproteins, this results in a large number of potential CTL targets and it is not known which protein would be the most relevant.

HSV infection is characterized by minimal presence of free virus. During latency and reactivation virus is mainly intracellular. Accordingly, recurrent disease is not prevented even by high levels of neutralizing antibodies and virus control depends on cell mediated immunity. In order to obtain protection by vaccination, it seems therefore desirable to induce not just an antibody response, but also CTL. An effective vaccine should prime CTL capable of acting as early as possible as soon as signs of reactivation of latent virus appear.

Previous studies have identified human CTL responses to various herpes simplex structural components such as glycoproteins gD, gB (Zarling et al. 1986), but the relevance of these CTL for virus clearance is not known. Moreover, such CTL were HLA class II restricted, and although expression of class II molecules is induced in keratinocytes during HSV replication, it may occur too late to prevent the appearance of lesions.

In order to identify the most important CTL target antigens for prophylatic or therapeutic vaccine purposes, the present inventors have taken into consideration the HSV replicative cycle. After primary infection and during reactivation from a latent state in neuronal ganglia, HSV is mostly intracellular, with minimal exposure to neutralizing antibodies. However, the beginning of viral protein synthesis inside a cell that harbours viral genome will generate viral protein fragments that will be presented by MHC molecules on the surface of the cell, making it a target for CTL of the appropriate specificity. The replication cycle of HSV lasts about 18-20 hours and involves an ordered expression of α or immediate early (IE) β or early (E) and γ or late (L) gene products. Therefore early CTL attack and consequent lysis of the infected cells prior to late structural gene expression could prevent new virions being made and therefore prevent spread of the virus to neighbouring cells. In order to be most useful, CTL should detect the very first viral proteins that appear inside the cell after infection and reactivation.

We have analyzed the specificity of human HSV specific CTL towards immediate early viral protein ICP4 (Infected Cell Protein 4). First, we investigated the CTL response in peripheral blood mononuclear cells (PBMC) from patients with herpetic genital lesions of varying clinical severity. We used autologous HSV-2 infected lymphoblasts as stimulators to induce HSV-2 specific CTL in limiting dilution cultures. HSV-2 specific responses were found in PBMC samples obtained days to weeks after the occurrence of lesions. The frequency of HSV-2 specific CTL ranged between 20 and 167 per million PBMC.

Using vaccinia virus recombinant ICP4.VV the gene product was expressed in EBV transformed lymphoblastoid target cells for cytotoxicity assays. The recombinant infected target cells were recognized by a fraction of HSV-2 specific CTL induced by in vitro restimulation with HSV-2 infected lymphoblasts. This IE protein constitutes therefore a candidate component for HSV vaccines aimed at inducing CTL mediated immunity.

The present invention is therefore, directed towards an immediate early HSV-2-viral protein ICP4 that is recognised by cytolytic T lymphocyte (CTL) in humans. In particular an ICP4 having substantially the sequence as shown in ID Sequence No.1

(protein sequence). The term substantially means at least 85% homologous, preferably 90 to 95% homologous, more preferably greater than 95% homologous.

Accordingly, the present invention provides a vaccine composition, for therapeutically or prophylactically treating HSV infections, comprising HSV-2, immediate early protein ICP4 or an immunologically active fragment thereof. The ICP4 protein may be expressed as a fusion protein or on a carrier such as a Hepatitis B surface antigen, or presented by a live bacterial carrier, such as listeria, shigella, BCG or Salmonella. Alternatively, the protein may be presented as in a live viral vector, such as vaccina, adenovirus or poliovirus. Alternatively the protein may be incorporated into an HSV light particle, as described in British patent application No. 91147140.0 and 9109763.4. (published: WO 92/13943 and PCT GB92/00824).

Such forms of presentation of ICP4 form part of the invention. A preferred embodiment of the invention is a vaccinia recombinant which expresses an HSV-2 ICP4 protein or an immunologically active fragment thereof.

This is the first medical use ascribed to this protein, and accordingly in one aspect of the invention there is provided HSV-2 ICP4 for use in medicine.

ICP4 of HSV-1 has been shown to be a target of CTL in CH3/HeN mice (H-2^k) (Martin, S. et al. 1990. Murine cytotoxic T lymphocytes specific for herpes simplex type 1 recognize the immediate early protein ICP4 but not ICP0. J. Gen. Virol. 71:2391-2399).

As used herein, an immunological fragment of ICP4 is a portion of the protein which is capable of eliciting a functional immunological response.

A further aspect of the invention provides a process for the preparation of the ICP4 HSV-2 protein or an immunogenic derivative thereof, which process comprises expressing DNA encoding said protein or derivative thereof in a recombinant host cell and recovering the product, and thereafter, optionally, preparing a derivative thereof.

A DNA molecule comprising such coding sequence eg as shown in ID Sequence No.2 or a fragment thereof forms a further aspect of the invention and can be synthesized by standard DNA synthesis techniques, such as by enzymatic ligation as described by D.M. Roberts et al in Biochemistry 1985, 24, 5090-5098, by chemical synthesis, by *in vitro* enzymatic polymerization, or by a combination of these techniques.

Enzymatic polymerisation of DNA may be carried out *in vitro* using a DNA polymerase such as DNA polymerase I (Klenow fragment) in an appropriate buffer containing the nucleoside triphosphates dATP, dCTP, dGTP and dTTP as required at a temperature of 10°-37°C, generally in a volume of 50µl or less. Enzymatic ligation of DNA fragments may be carried out using a DNA ligase such as T4 DNA ligase in an appropriate buffer, such as 0.05M Tris (pH 7.4), 0.01M $MgCl_2$, 0.01M dithiothreitol, 1mM spermidine, 1mM ATP and 0.1mg/ml bovine serum albumin, at a temperature of 4°C to ambient, generally in a volume of 50µl or less. The chemical synthesis of the DNA polymer or fragments may be carried out by conventional phosphotriester, phosphite or phosphoramidite chemistry, using solid phase techniques such as those described in 'Chemical and Enzymatic Synthesis of Gene Fragments - A Laboratory Manual' (ed. H.G. Gassen and A. Lang), Verlag Chemie, Weinheim (1982),or in other scientific publications, for example M.J. Gait, H.W.D. Matthes, M. Singh, B.S. Sproat, and R.C. Titmas, Nucleic Acids Research, 1982, 10, 6243; B.S. Sproat and W. Bannwarth, Tetrahedron Letters, 1983, 24, 5771; M.D. Matteucci and M.H Caruthers, Tetrahedron Letters, 1980, 21, 719; M.D. Matteucci and M.H. Caruthers, Journal of the American Chemical Society, 1981, 103, 3185; S.P. Adams et al., Journal of the American Chemical Society,1983, 105, 661; N.D. Sinha, J. Biernat, J. McMannus, and H. Koester, Nucleic Acids Research, 1984, 12, 4539; and H.W.D. Matthes et al., EMBO Journal, 1984, 3, 801.

Alternatively, the coding sequence can be derived from HSV-2 mRNA, using known techniques (e.g. reverse transcription of mRNA to generate a complementary cDNA strand), and commercially available cDNA kits.

The invention is not limited to the specifically disclosed sequence, but includes all molecules coding for the protein or an immunogenic derivative thereof, as described above.

DNA polymers which encode mutants of the protein of the invention may be prepared by site-directed mutagenesis of the cDNA which codes for the protein by conventional methods such as those described by G. Winter et al in Nature 1982, **299**, 756-758 or by Zoller and Smith 1982; Nucl. Acids Res., **10**, 6487-6500, or deletion mutagenesis such as described by Chan and Smith in Nucl. Acids Res., 1984, **12**, 2407-2419 or by G. Winter *et al* in Biochem. Soc. Trans., 1984, **12**, 224-225.

The process of the invention may be performed by conventional recombinant techniques such as described in Maniatis *et. al.*, Molecular Cloning - A Laboratory Manual; Cold Spring Harbor, 1982-1989.

In particular, the process may comprise the steps of:

i) preparing a replicable or integrating expression vector capable, in a host cell, of expressing a DNA polymer comprising a nucleotide sequence that encodes said HSV-2 ICP4 protein or an immunogenic derivative thereof;

ii) transforming a host cell with said vector;

iii) culturing said transformed host cell under conditions permitting expression of said DNA polymer to produce said protein; and

iv) recovering said protein.

The term 'transforming' is used herein to mean the introduction of foreign DNA into a host cell by transformation, transfection or infection with an appropriate plasmid or viral vector using e.g. conventional techniques as described in Genetic Engineering; Eds. S.M. Kingsman and A.J. Kingsman; Blackwell Scientific Publications; Oxford, England, 1988. The term 'transformed' or 'transformant' will hereafter apply to the resulting host cell containing and expressing the foreign gene of interest.

The expression vector is novel and also forms part of the invention.

The replicable expression vector may be prepared in accordance with the invention, by cleaving a vector compatible with the host cell to provide a linear DNA segment having an intact replicon, and combining said linear segment with one or more DNA molecules which, together with said linear segment encode the desired product, such as the DNA polymer encoding the 16 kDa protein, or fragments thereof, under ligating conditions.

Thus, the DNA polymer may be preformed or formed during the construction of the vector, as desired.

The choice of vector will be determined in part by the host cell, which may be prokaryotic or eukaryotic. Suitable vectors include plasmids, bacteriophages, cosmids and recombinant viruses.

The preparation of the replicable expression vector may be carried out conventionally with appropriate enzymes for restriction, polymerisation and ligation of the DNA, by procedures described in, for example, Maniatis *et al* cited above.

The recombinant host cell is prepared, in accordance with the invention, by transforming a host cell with a replicable expression vector of the invention under transforming conditions. Suitable transforming conditions are conventional and are described in, for example, Maniatis et al cited above, or "DNA Cloning" Vol. II, D.M. Glover ed., IRL Press Ltd, 1985.

The choice of transforming conditions is determined by the host cell. Thus, a bacterial host such as *E. coli* may be treated with a solution of $CaCl_2$ (Cohen *et al*, Proc. Nat. Acad. Sci., 1973, 69, 2110) or with a solution comprising a mixture of .bCl, $MnCl_2$, potassium acetate and glycerol, and then with

3-[N-morpholino]-propane-sulphonic acid, RbCl and glycerol. Mammalian cells in culture may be transformed by calcium co-precipitation of the vector DNA onto the cells. The invention also extends to a host cell transformed with a replicable expression vector of the invention.

Culturing the transformed host cell under conditions permitting expression of the DNA polymer is carried out conventionally, as described in, for example, Maniatis **et al** and "DNA Cloning" cited above. Thus, preferably the cell is supplied with nutrient and cultured at a temperature below 45°C.

The product is recovered by conventional methods according to the host cell. Thus, where the host cell is bacterial, such as **E. coli** it may be lysed physically, chemically or enzymatically and the protein product isolated from the resulting lysate. Where the host cell is mammalian, the product may generally be isolated from the nutrient medium or from cell free extracts. Conventional protein isolation techniques include selective precipitation, absorption chromatography, and affinity chromatography including a monoclonal antibody affinity column.

Alternatively, the expression may be carried out in insect cells using a suitable vector such as the Baculovirus. In a particular aspect of this invention, the protein is expressed in Lepidoptera cells to produce immunogenic polypeptides. For expression of the protein in Lepidoptera cells, use of a baculovirus expression system is preferred. In such system, an expression cassette comprising the protein coding sequence, operatively linked to a baculovirus promoter, typically is placed into a shuttle vector. Such vector contains a sufficient amount of bacterial DNA to propagate the shuttle vector in *E. coli* or some other suitable prokaryotic host. Such shuttle vector also contains a sufficient amount of baculovirus DNA flanking the desired protein coding sequence so as to permit recombination between a wild-type baculovirus and the heterologous gene. The recombinant vector is then cotransfected into Lepidoptera cells with DNA from a wild-type baculovirus. The recombinant baculoviruses arising from homologous recombination are then selected and plaque purified by standard techniques. See Summers et al., **TAES Bull** (Texas Agricultural Experimental Station Bulletin) **NR 1555**, May, 1987.

A process for expressing the CS protein in insect cells is described in detail in USSN 287,934 of SmithKline RIT (WO/US 89/05550).

Production in insect cells can also be accomplished by infecting insect larvae. For example, the protein can be produced in *Heliothis virescens* caterpillars by feeding the recombinant baculovirus of the invention along with traces of wild type baculovirus and then extracting the protein from the hemolymph after about two days. See, for example, Miller *et al.*, PCT/WO88/02030.

The novel protein of the invention may also be expressed in yeast cells as described for the CS protein in EP-A-0 278 941.

Vaccina constructs can be made by methods well known in the art, see for example European Patent Application EP-083-286 Health Research Inc., Inventors Paoletti and Panicali. The construction of such a vaccinia construct is presented in more detail in the examples.

ICP4 has been shown by the present inventors, to be recognised by human HSV specific CTL induced by in vitro stimulation of PBMC (peripheral blood mononuclear cells) with HSV-2 infected cells. By using infected cells, as stimulator cells in vitro, viral epitopes which are synthesized in the cytoplasm, are preferentially presented by class I molecules. Thus the spectrum of effector cells stimulated in vitro by this approach will include both class I and class II restricted T cells.

This is in contrast with stimulation of primed PBMC using inactivated free virus, known to preferentially induce class II restricted effector CTLs, as the virus enters antigen presenting cells by endocytosis and is processed by the class II pathway. Neo-synthesis of antigen does not occur, and class I restricted presentation is less likely to occur.

The antigenic specificity of human CTL responses to HSV is highly relevant for an effective subunit vaccine, since HSV infection is characterised by the ability to establish latency and reactivate periodically. During latency and reactivation there is minimal exposure of free virus to antibodies as the virus exists mainly intracellularly.

In order to maximise the protective ability of a vaccine according to the invention, the vaccine may also preferably contain one or more other HSV proteins, other immediate early, early or late proteins capable of stimulating a CTL response in humans, such as gD or gC, Vmw65, RR2, ICPO or ICP27. In particular, the vaccine may advantageously contain a truncated gD derivative from HSV-2 as described in EP 139 417 B. Also the vaccine may contain HSV-1 proteins or cocktails of variants of the same proteins where they exist.

Also the vaccine may contain HSV-1 proteins or cocktails of variants of the same proteins where they exist.

The vaccine of the present invention will preferably be adjuvanted. Known adjuvants will include aluminium salts, mycobacterium derived antigens such as Freunds complete or incomplete adjuvants, and muramyldipeptide (MDP) and derivatives, saponin type adjuvants such as QS21 (US Patent No 5057540) and the like. A particularly preferred adjuvant preparation is 3-0-de-acylated monophosphoryl lipid A (MPL) which is commercially available from Ribi Immunochem and may be prepared according to the method of GB 2220211, or QS21 commercially available from Cambridge Biotech.

In such cases MPL and/or QS21 will be present in the range 10μg - 100μg, and preferably 25 - 50 μg per dose. The vaccine containing MPL or QS21 will typically be presented on alum or in an oil in water emulsion.

Additionally, the vaccine could be in various liposome forms including Novasomes.

Vaccine preparation is generally described in New Trends and Developments in Vaccines, edited by Voller et al., University Park Press, Baltimore, Maryland, U.S.A. 1978. Encapsulation within liposomes is described, for example, by Fullerton, U.S. Patent 4,235,877. Conjugation of proteins to macromolecules is disclosed, for example, by Likhite, U.S. Patent 4,372,945 and by Armor et al., U.S. Patent 4,474,757.

The amount of protein in each vaccine dose is selected as an amount which induces an immunoprotective response without significant, adverse side effects in typical vaccinees. Such amount will vary depending upon which specific immunogen is employed and how it is presented. Generally, it is expected that each dose will comprise 1-1000 μg of protein, preferably 2-100 μg, most preferably 4-40 μg. An optimal amount for a particular vaccine can be ascertained by standard studies involving observation of appropriate immune responses in subjects. Following an initial vaccination, subjects may receive one or several booster immunisation adequately spaced.

In addition to vaccination of persons susceptible to HSV infections, the pharmaceutical compositions of the present invention may be used to treat, immunotherapeutically, patients suffering from HSV infections, in order to prevent or significantly decrease recurrent herpes disease, frequency, severity and duration of episodes.

The rationale for immunotherapeutic use of the invention is that the frequency of HSV specific CTL, that exert an immune surveillance function against the virus, may physiologically decline with time after the last antigen-triggered expansion. Alternatively virus infection may not trigger a strong enough CTL response.

When low numbers of such CTL exist in the body, a reactivating HSV infection will have more chances to go through more rounds of viral replication before being detected by HSV specific T cells, resulting in larger clinically apparent herpetic lesions. However, if CTL levels are maintained at a given level by a suitable protocol of therapeutic vaccination, the time during which reactivating virus replicates unchecked will be kept to a minimum. This will have a beneficial effect in HSV infected individuals, eliminating or reducing the severity of clinically detectable recurrent lesions. This effect will be in addition to, and non exclusive of, the

advantage provided by the specificity of CTL for an immediate early antigen, as referred to above.

A suitable protocol of therapeutic vaccination may be defined as a pharmacologically acceptable amount of vaccine preparation administered at regular time intervals in HSV infected individuals, which results in elimination or reduced severity of previously occurring recurrent herpetic disease.

EXAMPLE 1: Expression of ICP4 (HSV-2) in vaccinia virus

1. CONSTRUCTION OF AN INSERTION PLASMID TO OBTAIN A RECOMBINANT VACCINIA VIRUS EXPRESSING THE ICP4 PROTEIN OF HSV-2 (STRAIN HG52)

- We received the plasmid pBB17 from B.C. Barnett (MRC Virology Unit, Institute of Virology, Church Street, Glasgow G11 5JR). It's a pUC19 derived plasmid with the Kpn 1a-Hind IIIk fragment of HSV-2 genome and, therefore, carries the 5' terminal sequence of the ICP4 gene. The pBB17 was digested by Nru I and Asp718 restriction enzymes and a fragment of 3355 bp containing the 5' part of the gene (except for the first 67 bp) was isolated.

- The beginning of the gene was reconstituted by a PCR fragment flanked by Eco RI and Nru I restriction sites.

- Preparation of the PCR fragment:

5' primer = CMo Seq 21: 5' CGC TAG AAT TCA GAT CTG CCA CCA TGT CGG CGG AGC AGC GG 3'

3' primer = CMo Seq 23: 5' CGC TAG GTA CCC CGC CGG TCG TCT CC 3'

Template: pBB17

The reaction was achieved according to the Perkin Elmer Cetus GeneAmp PCR reagent kit (except for the amount of template: 1µg in our case)

The reaction conditions were:

2 min at 94° C)
2 min at 55° C) 25 cycles
2 min at 72° C)
15 min at 72°C;→15°C

So, a fragment of about 166bp was obtained. It was digested by Eco RI and Asp 718 restriction enzymes to give a 140 bp fragment. This fragment was then isolated and ligated into pUC18 cut by Eco RI and Asp718 to obtain pRit14068.

- The pRit14068 was cut by Nru I and Asp718 restriction enzymes and ligated with the 3355 bp fragment (derived from pBB17: see above) to obtain the pRit13643.

- We received the plasmid "Bam g" (it is the Bam HI g fragment of HSV-2 genome cloned in PAT153) from D.J. McGeoch (MRC Virology Unit. Institute of Virology, Church Street, Glasgow G11 5JR).
  This plasmid carries the 3' terminal sequence of the ICP4 gene. It was digested by Asp718 and Sph I restriction enzymes and a fragment of 1733 bp containing the 3' part of the gene was isolated.

  The pRit13643 was cut by Asp718 and Sph I, and ligated with the 1733 bp fragment to obtain pRit13644.

- The vaccinia virus expression vector used was the pULB5212. We have received this plasmid from F. Bex (ULB. Rhode-St-Genèse). It is a derivative of pSC11 (vaccinia virus expression vector: co-expression of β-galactosidase provides visual screening of recombinant virus plaques. S. Chakrabarti and al. Molecular and Cellular Biology, Dec. 1985, p 3403-3409) with a multiple cloning sites polylinker inserted into Sma I. The sequence of this polylinker is 5'AGA TCT GGT ACC GCA TGC CCC 3'. This plasmid was cut by Bgl II and Sph I restriction enzymes and ligated to the Bgl II-Asp718 fragment of 3433 bp (isolated from pRit13643) and the Asp718-Sph I fragment of 1733 bp (isolated from "Bam g") to obtain pRit13645.

- A fragment of 1111 bp was isolated from the pRit13644 digested by Asp718 and Sma I. This fragment was inserted in pUC18 cut by Asp718 and Hinc II. The resulting plasmid was the pRit14069. From this plasmid, it was possible to re-isolate the 3' part of ICP4 gene as a fragment Asp718-Sph I (1125 bp) and to exchange it with the fragment Asp718-Sph I (1733 bp) of pRit13645 to obtain pRit14070. This last step was added to eliminate the "a" sequences present in the pRit13645.

- The pRit 14070 have been used to transfect vaccinia virus (WR) infected CV1 cells and so to achieve a recombinant vaccinia virus expressing ICP4 protein. (For the method see: Sekhar Chakrabarti, Kathleen Brechling, and Bernard Moss. 1985. Vaccinia virus expresssion vector: co-expression of β-Galactosidase provides visual screening of recombinant virus plaques. Mol. Cell. Biol. 5: 3403-3409).

This method was performed with success and several recombinant vaccinia virus expression ICP4 have been obtained. Expression of ICP4 protein was detected by Western Blot in BHK21 cell lysates infected with ICP4 recombinant viccinia virus.

Note: • WR: ATCC VR - 119 vaccinia virus.
• CVI: Kidney, African Green Monkey cells : ATCC CCL70.

FIG: A.) Scheme of the construction of a vaccinia virus insertion vector
B.) containing the ICP4 gene of HSV2.
C. Expression of ICP4-HSV2 by a recombinant vaccinia virus. Detection by western blot.

EXAMPLE 2: Recognition of ICP4 by human CTL

Materials and methods:

Patients. Blood samples from patients attending the sexually transmitted disease clinic of the Topical Medicine Institute, Antwerp, were collected by venipuncture into heparinised tubes. Patients had genital herpetic lesions of varying clinical severity and differed in recurrent disease patterns. One asymptomatic sexual partner of a patient with recurrent disease was included in the study.

Viruses. Herpes simplex virus. The HG52 strain of herpes simplex virus type 2(HSV-2) used in these experiments was kindly provided by Prof. Subak-Sharpe (MRC, Glasgow, U.K.). The virus was grown in BHK21 cells infected at a multiplicity of infection (m.o.i.) of 0.003 plaque forming units (p.f.u.) per cell. The cells were harvested at 5-7 days after infection, disrupted by freezing/thawing and sonicated. The virus titre was determined by plaque assay on BHK21 cells.

ICP4 vaccinia recombinant was produced as herein described.

Medium. PBMC cultures were grown in RPMI 1640 (Gibco, Ghent, Belgium) supplemented with 10% (v/v) heat inactivated foetal calf serum (FCS)(Flow laboratories, Irwine, Scotland), 2 x 10^{-3} M L-glutamine, 100 IU/mL penicillin, 100 µg/mL streptomycin, 5 x 10^{-5} M mercaptoethanol, 1% MEM non-essential amino acids (Gibco), 1 x 10^{-3} M sodium pyruvate MEM (Gibco).

Cells. Peripheral blood mononuclear cells (PBMC) were obtained from blood by separation on a Lymphoprep (Nycomed, Oslo, Norway) density gradient (Boyum, A. (1968) Scand. J. Clin. Lab. Invest. 21, Suppl. 97, 77). PBMC were frozen in 10% DMSO 90% FCS (v/v) and thawed just before use as responder cells.

An aliquot of PBMC from each patient was used to derive lymphoblastoid cell lines (LCL) by transformation with Epstein-Barr virus (EBV obtained from culture

supernatants of the persistently infected marmoset cell line B-95.8, as described (Walls, E.V. and Crawford, D.H. (1987)) Generation of human B lymphoblastoid cell lines using Epstein - Barr virus. In : Lymphocytes, a practical approach, Klaus, G.G.B. (editor) pp.149 - 162. The LCL were used as target cells in cytotoxicity assays.

PHA-activated lymphoblasts for use as stimulator cells were prepared by culturing 5 x 10^6 PBMC with 4 µg/mL PHA-P (Sigma) for 72 hours at 37°C and with 5 U.mL rIL2 (Boehringer) for 7 more days at 37°C. The lymphoblasts were then infected with HSV-2 (m.o.i.= 10) for 16 hours at 37°C and treated with 1% formaldehyde in PBS for 20 min at 4°C.

Limiting dilution cultures. PBMC were thawed and distributed into 96-well round-bottom plates. The number of responder cells per well ranged between 10^3 and 4 x 10^4 and 24 to 32 wells were set up for each input cell concentration. Autologous stimulator cells (5 x 10^4/well) were added to all wells. Control wells without responder cells were included. Cultures received 1 U/ml rIL-2 and 5% (v/v) PHA-blast supernatant at the onset, and were fed with 5U/ml rIL-2 and 5% (v/v) PHA-blast supernatant every 4-6 days. Equal aliquots from each individual culture were tested on day 14-21 in a chromium release assay against 3 different target cell types; autologous LCL infected with HSV-2, psC11.VV and ICP4.VV.

Cytotoxicity assays. LCL target cells were infected with HSV-2, psC11.VV or ICP4.VV (m.o.i.=10) for 1 hour at 37°C, washed and labelled with 500 µCi of ^{51}Cr (Medgenix, Fleurus, Belgium) for 1 hour at 37°C. Target cells were then washed twice, incubated on ice for 30 min, washed once and 2 x 10^3 cells per well were distributed into the wells containing responder cells and control wells containing medium or Triton X-100 3% in water (spontaneous release and maximum release, respectively). Effector and target cell mixtures were incubated for 4 hours at 37°C in a total of 200 µL, then 100 µL of supernatant were harvested and released ^{51}Cr counted. Results were expressed as % specific lysis according to the formula:

$$\% \text{ specific lysis} = \frac{(\text{experimental} - \text{spontaneous release})}{(\text{total} - \text{spontaneous release})} \times 100$$

CTL frequency determinations. Responder frequencies were calculated using the maximum likelihood method described by Fazekas de St. Groth (Fazekas de St. Groth, S. (1982). The evaluation of limiting dilution assays. J. Imm. Meth. 49:R11-R23). For each target cell type, wells were scored as positive if the % specific lysis was higher than the cut-off value defined as the average of control wells without responder cells + 3 standard deviations. The frequency estimates of HSV-2 and ICP4

specific CTL were obtained after exclusion of any wells that scored positive on control targets (psC11.VV infected).

Recognition of ICP4 of HSV-2 by HSV-2 specific CTL. In order to evaluate the role of ICP4 in HSV-2 recognition by human CTL, limiting dilution cultures of PBMC from genital herpes patient, stimulated in vitro with HSV-2 infected autologous lymphoblasts, were split 4-ways and 3 aliquots from each well tested on autologous LCL infected with HSV-2, psC11.VV or ICP4.VV (Table I). Out of 13 patients tested, 4 (I18, I39, I44 and S12) had high frequencies of effectors that lysed pSC11.VV infected target cells, and were therefore not considered. In 5 patients (I06, I26, I27, I46 and I50) the frequency of HSV-2 specific CTL ranged between 20 and 167 per million PBMC, while the remaining 4 patients had frequencies of HSV-2 specific CTL lower than 20 per million PBMC.

Out of 5 patients with high frequencies of HSV-2 specific CTL, three (I06, I27 and I46) had ICP4 specific CTL (frequencies of 26, 23 and 29 CTL per million PBMC). In patient I46, ICP4 appeared to be the main HSV-2 antigen recognized by CTL.

Table I.

Recognition of ICP4 of HSV-2 by human CTL from patients with genital herpes.

Patient	pSC11.VV[a]	HSV-2	ICP4.VV
I06	8[b]	63	26
I18	20	125	26
I26	8	167	4
I27	14	29	23
I35	ND[c]	13	3
I39	125	67	42
I40	5	3	4
I41	ND	ND	ND
I44	27	ND	ND
I46	10	26	29
I50	12	20	17
S02	3	3	6
S04	15	ND	3
S12	63	ND	5

a Virus used to infect autologous target cells.

b Number of specific CTL per million PBMC.

c ND: not determinable.

We show herein for the first time that ICP4 HSV-2 is recognized by human HSV specific CTL induced by in vitro stimulation of PBMC with HSV-2 infected cells. ICP4 of HSV-2 is a 183 Kdalton polypeptide coded by one of the five alpha genes that are expressed first upon infection, and reaches peak synthesis at 2-4 hours. ICP4 has regulatory functions. In a subset of 5 patients with frequencies of HSV-2 specific CTL ranging between 20 and 167 per million PBMC, 3 patients had 23-29 ICP4 specific CTL per million PBL. These frequencies are calculated after exclusion of all cultures scoring positive on control target cells, and constitute therefore minimal estimates.

These results show that a fraction of the human response to herpes simplex virus is directed against a non-virion polypeptide.

Sequence ID 1

Protein sequence of Icp 4 from HSV 2

1 MSAEQRKKKK TTTTTQGRGA EVAMADEDGG RLRAAAETTG GPGSPDPADG

51 PPPTPNPDRR PAARPGFGWH GGPEENEDEA DDAAADADAD EAAPASGEAV

101 DEPAADGVVS PRQLALLASM VDEAVRTIPS PPPERDGAQE EAARSPSPPR

151 TPSMRADYGE ENDDDDDDDD DDDRDAGRWV RGPETTSAVR GAYPDPMASL

201 SPRPPAPRRH HHHHHHRRRR APRRRSAASD SSKSGSSSSA SSASSSASSS

251 SSASASSSDD DDDDDAARAP ASAADHAAGG TLGADDEEAG VPARAPGAAP

301 RPSPPRAEPA PARTPAATAG RLERRRARAA VAGRDATGRF TAGRPRRVEL

351 DADAASGAFY ARYRDGYVSG EPWPGAGPPP PGRVLYGGLG DSRPGLWGAP

401 EAEEARARFE ASGAPAPVWA PELGDAAQQY ALITRLLYTP DAEAMGWLQN

451 PRVAPGDVAL DQACFRISGA ARNSSSFISG SVARAVPHLG YAMAAGRFGW

501 GLAHVAAAVA MSRRYDRAQK GFLLTSLRRA YAPLLARENA ALTGARTPDD

551 GGDANRHDGD DARGKPAAAA APLPSAAASP ADERAVPAGY GAAGVLAALG

601 RLSAAPASAP AGADDDDDDD GAGGGGGGRR AEAGRVAVEC LAACRGILEA

651 LAEGFDGDLA AVPGLAGARP AAPPRPGPAG AAAPPHADAP RLRAWLRELR

701 FVRDALVLMR LRGDLRVAGG SEAAVAAVRA VSLVAGALGP ALPRSPRLLS

751 SAAAAAADLL FQNQSLRPLL ADTVAAADSL AAPASAPREA RKRKSPAPAR

801 APPGGAPRPP KKSRADAPRP AAAPPAGAAP PAPPTPPPRP PRPAALTRRP

851 AEGPDPQGGW RRQPPGPSHT PAPSAAALEA YCAPRAVAEL TDHPLFPAPW

901 RPALMFDPRA LASLAARCAA PPPGGAPAAF GPLRASGPLR RAAAWMRQVP

951 DPEDVRVVIL YSPLPGEDLA AGRAGGGPPP EWSAERGGLS CLLAALGNRL

1001 CGPATAAWAG NWTGAPDVSA LGAQGVLLLS TRDLAFAGAV EFLGLLAGAC

1051 DRRLIVVNAV RAADWPADGP VVSRQHAYLA CEVLPAVQCA VRWPAARDLR

1101 RTVLASGRVF GPGVFARVEA AHARLYPDAP PLRLCRGANV RYRVRTRFGP

1151 DTLVPMSPRE YRRAVLPALD GRAAASGAGD AMAPGAPDFC EDEAHSHRAC

1201 ARWGLGAPLR PVYVALGRDA VRGGPAELRG PRREFCARAL LEPDGDAPPL

1251 VLRDDADAGP PPQIRWASAA GRAGTVLAAA GGGVEVVGTA AGLATPPRRE

1301 PVDMDAELED DDDGLFGE*

Sequence ID 2

Dna sequence containing the coding region of Icp4 from Hsv2

```
    ATGTCGGCGGAGCAGCGGAAGAAGAAGAAGACGACGACGACGACGCAGGGCCGCGGGGCC
248 --+---------+---------+---------+---------+---------+---------+------- 307
    TACAGCCGCCTCGTCGCCTTCTTCTTCTTCTGCTGCTGCTGCGTCCCGGCGCCCCGG

    GAGGTCGCGATGGCGGACGAGGACGGGGGACGTCTCCGGGCCGCGGCGGAGACGACCGGC
308 --+---------+---------+---------+---------+---------+---------+------- 367
    CTCCAGCGCTACCGCCTGCTCCTGCCCCCTGCAGAGGCCCGGCGCCGCCTCTGCTGGCCG

    GGCCCCGGATCTCCGGATCCAGCCGACGGACCGCCGCCCACCCCGAACCCGGACCGTCGC
368 --+---------+---------+---------+---------+---------+---------+------- 427
    CCGGGGCCTAGAGGCCTAGGTCGGCTGCCTGGCGGCGGGTGGGGCTTGGGCCTGGCAGCG

    CCCGCCGCGCGGCCCGGGTTCGGGTGGCACGGTGGGCCGGAGGAGAACGAAGACGAGGCC
428 --+---------+---------+---------+---------+---------+---------+------- 487
    GGGCGGCGCGCCGGGCCCAAGCCCACCGTGCCACCCGGCCTCCTCTTGCTTCTGCTCCGG

    GACGACGCCGCCGCCGATGCCGATGCCGACGAGGCGGCCCCGGCGTCCGGGGAGGCCGTC
488 --+---------+---------+---------+---------+---------+---------+------- 547
    CTGCTGCGGCGGCGGCTACGGCTACGGCTGCTCCGCCGGGGCCGCAGGCCCCTCCGGCAG

    GACGAGCCTGCCGCGGACGGCGTCGTCTCGCCGCGGCAGCTGGCCCTGCTGGCCTCGATG
548 --+---------+---------+---------+---------+---------+---------+------- 607
    CTGCTCGGACGGCGCCTGCCGCAGCAGAGCGGCGCCGTCGACCGGGACGACCGGAGCTAC

    GTGGACGAGGCCGTTCGCACGATCCCGTCGCCCCCCCCGGAGCGCGACGGCGCGCAAGAA
608 --+---------+---------+---------+---------+---------+---------+------- 667
    CACCTGCTCCGGCAAGCGTGCTAGGGCAGCGGGGGGGGCCTCGCGCTGCCGCGCGTTCTT

    GAAGCGGCCCGCTCGCCTTCTCCGCCGCGGACCCCCTCCATGCGCGCCGATTATGGCGAG
668 --+---------+---------+---------+---------+---------+---------+------- 727
    CTTCGCCGGGCGAGCGGAAGAGGCGGCGCCTGGGGGAGGTACGCGCGGCTAATACCGCTC

    GAGAACGACGACGACGACGACGACGACGATGACGACGACCGCGACGCGGGCCGCTGGGTC
728 --+---------+---------+---------+---------+---------+---------+------- 787
    CTCTTGCTGCTGCTGCTGCTGCTGCTACTGCTGCTGGCGCTGCGCCCGGCGACCCAG

    CGCGGACCGGAGACGACGTCCGCGGTCCGCGGGGCGTACCCGGACCCCATGGCCAGCCTG
788 --+---------+---------+---------+---------+---------+---------+------- 847
    GCGCCTGGCCTCTGCTGCAGGCGCCAGGCGCCCCGCATGGGCCTGGGGTACCGGTCGGAC

    TCGCCGCGACCCCCGGCGCCCCGCCGACACCACCACCACCACCACCACCGCCGCCGGCGC
848 --+---------+---------+---------+---------+---------+---------+------- 907
    AGCGGCGCTGGGGGCCGCGGGGCGGCTGTGGTGGTGGTGGTGGTGGCGGCGGCCGCG

    GCCCCCCGCCGGCGCTCGGCCGCCTCTGACTCATCAAAATCCGGATCCTCGTCGTCGGCG
908 --+---------+---------+---------+---------+---------+---------+------- 967
    CGGGGGGCGGCCGCGAGCCGGCGGAGACTGAGTAGTTTTAGGCCTAGGAGCAGCAGCCGC

    TCCTCCGCCTCCTCCTCCGCCTCCTCCTCCTCGTCTGCATCCGCCTCCTCGTCTGACGAC
968 --+---------+---------+---------+---------+---------+---------+------- 1027
    AGGAGGCGGAGGAGGAGGCGGAGGAGGAGGAGCAGACGTAGGCGGAGGAGCAGACTGCTG
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     GACGACGACGACGACGCCGCCCGCGCCCCCGCCAGCGCCGCAGACCACGCCGCGGGCGGG
1028 --+---------+---------+---------+---------+---------+------- 1087
     CTGCTGCTGCTGCTGCGGCGGGCGCGGGGGCGGTCGCGGCGTCTGGTGCGGCGCCCGCCC

     ACCCTCGGCGCGGACGACGAGGAGGCGGGGGTGCCCGCGAGGGCCCCGGGGGCGGCGCCC
1088 --+---------+---------+---------+---------+---------+------- 1147
     TGGGAGCCGCGCCTGCTGCTCCTCCGCCCCCACGGGCGCTCCCGGGGCCCCCGCCGCGGG

     CGGCCGAGCCCGCCCAGGGCCGAGCCCGCCCCGGCCCGGACCCCCGCGGCGACCGCGGGC
1148 --+---------+---------+---------+---------+---------+------- 1207
     GCCGGCTCGGGCGGGTCCCGGCTCGGGCGGGGCCGGGCCTGGGGGCGCCGCTGGCGCCCG

     CGCCTGGAGCGCCGCCGGGCCCGCGCGGCGGTGGCCGGCCGCGACGCCACGGGCCGCTTC
1208 --+---------+---------+---------+---------+---------+------- 1267
     GCGGACCTCGCGGCGGCCCGGGCGCGCCGCCACCGGCCGGCGCTGCGGTGCCCGGCGAAG

     ACGGCCGGGCGGCCCCGGCGGGTCGAGCTGGACGCCGACGCGGCCTCCGGCGCCTTCTAC
1268 --+---------+---------+---------+---------+---------+------- 1327
     TGCCGGCCCGCCGGGGCCGCCCAGCTCGACCTGCGGCTGCGCCGGAGGCCGCGGAAGATG

     GCGCGCTACCGCGACGGGTACGTCAGCGGGGAGCCGTGGCCCGGGGCCGGCCCCCCGCCC
1328 --+---------+---------+---------+---------+---------+------- 1387
     CGCGCGATGGCGCTGCCCATGCAGTCGCCCCTCGGCACCGGGCCCCGGCCGGGGGGCGGG

     CCGGGGCGCGTGCTGTACGGCGGGCTGGGCGACAGCCGCCCCGGCCTCTGGGGGGCGCCC
1388 --+---------+---------+---------+---------+---------+------- 1447
     GGCCCCGCGCACGACATGCCGCCCGACCCGCTGTCGGCGGGGCCGGAGACCCCCCGCGGG

     GAGGCGGAGGAGGCGCGGGCCCGGTTCGAGGCCTCGGGCGCCCCGGCGCCCGTGTGGGCG
1448 --+---------+---------+---------+---------+---------+------- 1507
     CTCCGCCTCCTCCGCGCCCGGGCCAAGCTCCGGAGCCCGCGGGGCCGCGGGCACACCCGC

     CCCGAGCTGGGCGACGCGGCGCAGCAGTACGCCCTGATCACGCGGCTGCTGTACACGCCG
1508 --+---------+---------+---------+---------+---------+------- 1567
     GGGCTCGACCCGCTGCGCCGCGTCGTCATGCGGGACTAGTGCGCCGACGACATGTGCGGC

     GACGCGGAGGCGATGGGGTGGCTCCAGAACCCGCGCGTGGCGCCCGGGGACGTGGCGCTG
1568 --+---------+---------+---------+---------+---------+------- 1627
     CTGCGCCTCCGCTACCCCACCGAGGTCTTGGGCGCGCACCGCGGGCCCCTGCACCGCGAC

     GACCAGGCCTGCTTCCGGATCTCGGGCGCGGCGCGCAACAGCAGCTCCTTCATCTCCGGC
1628 --+---------+---------+---------+---------+---------+------- 1687
     CTGGTCCGGACGAAGGCCTAGAGCCCGCGCCGCGCGTTGTCGTCGAGGAAGTAGAGGCCG

     AGCGTGGCGCGGGCCGTGCCCCACCTGGGGTACGCCATGGCGGCGGGCCGCTTCGGCTGG
1688 --+---------+---------+---------+---------+---------+------- 1747
     TCGCACCGCGCCCGGCACGGGGTGGACCCCATGCGGTACCGCCGCCCGGCGAAGCCGACC

     GGCCTGGCGCACGTGGCGGCCGCCGTGGCCATGAGCCGCCGCTACGACCGCGCGCAGAAG
1748 --+---------+---------+---------+---------+---------+------- 1807
     CCGGACCGCGTGCACCGCCGGCGGCACCGGTACTCGGCGGCGATGCTGGCGCGCGTCTTC

     GGCTTCCTGCTGACCAGCCTGCGCCGCGCCTACGCGCCCCTGCTGGCGCGCGAGAACGCG
1808 --+---------+---------+---------+---------+---------+------- 1867
     CCGAAGGACGACTGGTCGGACGCGGCGCGGATGCGCGGGGACGACCGCGCGCTCTTGCGC

     GCGCTGACCGGGGCGCGAACCCCCGACGACGGCGGCGACGCCAACCGCCACGACGGCGAC
1868 --+---------+---------+---------+---------+---------+------- 1927
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     CGCGACTGGCCCCGCGCTTGGGGGCTGCTGCCGCCGCTGCGGTTGGCGGTGCTGCCGCTG

     GACGCCCGCGGGAAGCCCGCCGCCGCCGCCGCCCCGTTGCCGTCGGCGGCGGCGTCGCCG
1928 --+---------+---------+---------+---------+---------+---------+------- 1987
     CTGCGGGCGCCCTTCGGGCGGCGGCGGCGGCGGGGCAACGGCAGCCGCCGCCGCAGCGGC

     GCCGACGAGCGCGCGGTGCCCGCCGCTACGGCGCCGCGGGGGTGCTCGCCGCCCTGGGG
1988 --+---------+---------+---------+---------+---------+---------+------- 2047
     CGGCTGCTCGCGCGCCACGGGCGGCCGATGCCGCGGCGCCCCCACGAGCGGCGGGACCCC

     CGCCTGAGCGCCGCGCCCGCCTCCGCGCCGGCCGGGGCCGACGACGACGACGACGACGAC
2048 --+---------+---------+---------+---------+---------+---------+------- 2107
     GCGGACTCGCGGCGCGGGCGGAGGCGCGGCCGGCCCCGGCTGCTGCTGCTGCTGCTGCTG

     GGCGCCGGCGGTGGTGGCGGCGGCCGGCGCGCGGAGGCGGGCCGCGTGGCCGTGGAGTGC
2108 --+---------+---------+---------+---------+---------+---------+------- 2167
     CCGCGGCCGCCACCACCGCCGCCGGCCGCGCGCCTCCGCCCGGCGCACCGGCACCTCACG

     CTGGCCGCCTGCCGCGGGATCCTGGAGGCGCTGGCGGAGGGCTTCGACGGCGACCTGGCG
2168 --+---------+---------+---------+---------+---------+---------+------- 2227
     GACCGGCGGACGGCGCCCTAGGACCTCCGCGACCGCCTCCCGAAGCTGCCGCTGGACCGC

     GCCGTGCCGGGGCTGGCCGGAGCCCGGCCCGCCGCGCCCCCGCGCCCGGGGCCCGCGGGC
2228 --+---------+---------+---------+---------+---------+---------+------- 2287
     CGGCACGGCCCCGACCGGCCTCGGGCCGGGCGGCGCGGGGGCGCGGGCCCCGGGCGCCCG

     GCGGCCGCCCCGCCGCACGCCGACGCGCCCCGCCTGCGCGCCTGGCTGCGCGAGCTGCGG
2288 --+---------+---------+---------+---------+---------+---------+------- 2347
     CGCCGGCGGGGCGGCGTGCGGCTGCGCCGGGCGGACGCGCGGACCGACGCGCTCGACGCC

     TTCGTGCGCGACGCGCTGGTGCTGATGCGCCTGCGCGGGGACCTGCGCGTGGCCGGCGGC
2348 --+---------+---------+---------+---------+---------+---------+------- 2407
     AAGCACGCGCTGCGCGACCACGACTACGCGGACGCGCCCCTGGACGCGCACCGGCCGCCG

     AGCGAGGCCGCCGTGGCCGCCGTGCGCGCCGTGAGCCTGGTCGCCGGGGCCCTGGGCCCG
2408 --+---------+---------+---------+---------+---------+---------+------- 2467
     TCGCTCCGGCGGCACCGGCGGCACGCGCGGCACTCGGACCAGCGGCCCCGGGACCCGGGC

     GCGCTGCCGCGGAGCCCGCGCCTGCTGAGCTCCGCCGCCGCCGCCGCCGCGGACCTGCTC
2468 --+---------+---------+---------+---------+---------+---------+------- 2527
     CGCGACGGCGCCTCGGGCGCGGACGACTCGAGGCGGCGGCGGCGGCGGCGCCTGGACGAG

     TTCCAGAACCAGAGCCTGCGCCCCCTGCTGGCCGACACCGTCGCCGCGGCCGACTCGCTC
2528 --+---------+---------+---------+---------+---------+---------+------- 2587
     AAGGTCTTGGTCTCGGACGCGGGGGACGACCGGCTGTGGCAGCGGCGCCGGCTGAGCGAG

     GCCGCGCCCGCCTCCGCGCCGCGGGAGGCGCGCAAGCGCAAGAGCCCCGCCCCGGCCAGG
2588 --+---------+---------+---------+---------+---------+---------+------- 2647
     CGGCGCGGGCGGAGGCGCGGCGCCCTCCGCGCGTTCGCGTTCTCGGGGCGGGGCCGGTCC

     GCGCCGCCGGGCGGCGCCCCGCGCCCCCCGAAGAAGAGCCGCGCGGACGCCCCCCGCCCC
2648 --+---------+---------+---------+---------+---------+---------+------- 2707
     CGCGGCGGCCCGCCGCGGGGCGCGGGGGGCTTCTTCTCGGCGCGCCTGCGGGGGGCGGGG

     GCGGCCGCCCCTCCCGCGGGGGCCGCGCCCCCCGCCCCGCCGACGCCGCCGCCGCGGCCG
2708 --+---------+---------+---------+---------+---------+---------+------- 2767
     CGCCGGCGGGGAGGGCGCCCCCGGCGCGGGGGGCGGGGCGGCTGCGGCGGCGGCGCCGGC

     CCGCGCCCCGCGGCGCTGACCCGCCGGCCCGCCGAGGGCCCCGACCCGCAGGGCGGCTGG
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2768 --+---------+---------+---------+---------+---------+------- 2827
     GGCGCGGGGCGCCGCGACTGGGCGGCCGGGCGGCTCCCGGGGCTGGGCGTCCCGCCGACC

     CGCCGCCAGCCGCCGGGGCCCAGCCACACGCCGGCGCCCTCGGCCGCCGCCCTGGAGGCC
2828 --+---------+---------+---------+---------+---------+------- 2887
     GCGGCGGTCGGCGGCCCCGGGTCGGTGTGCGGCCGCGGGAGCCGGCGGCGGGACCTCCGG

     TACTGCGCCCCGCGGGCCGTGGCCGAGCTCACGGACCACCCGCTCTTCCCCGCGCCGTGG
2888 --+---------+---------+---------+---------+---------+------- 2947
     ATGACGCGGGGCGCCCGGCACCGGCTCGAGTGCCTGGTGGGCGAGAAGGGGCGCGGCACC

     CGCCCGGCCCTCATGTTCGACCCGCGCGCGCTGGCCTCGCTGGCCGCGCGCTGCGCCGCC
2948 --+---------+---------+---------+---------+---------+------- 3007
     GCGGGCCGGGAGTACAAGCTGGGCGCGCGACCGGAGCGACCGGCGCGCGACGCGGCGG

     CCGCCCCCCGGCGGCGCGCCCGCCGCCTTCGGCCCGCTGCGCGCCTCGGGCCCGCTGCGC
3008 --+---------+---------+---------+---------+---------+------- 3067
     GGCGGGGGGCCGCCGCGCGGGCGGCGGAAGCCGGGCGACGCGCGGAGCCCGGGCGACGCG

     CGCGCGGCGGCCTGGATGCGCCAGGTGCCCGACCCGGAGGACGTGCGCGTGGTGATCCTC
3068 --+---------+---------+---------+---------+---------+------- 3127
     GCGCGCCGCCGGACCTACGCGGTCCACGGGCTGGGCCTCCTGCACGCGCACCACTAGGAG

     TACTCGCCGCTGCCGGGCGAGGACCTGGCCGCGGGCCGCGCCGGGGGCGGGCCCCCCCCG
3128 --+---------+---------+---------+---------+---------+------- 3187
     ATGAGCGGCGACGGCCCGCTCCTGGACCGGCGCCCGGCGCGGCCCCCGCCCGGGGGGGGC

     GAGTGGTCCGCCGAGCGCGGCGGGCTGTCCTGCCTGCTGGCGGCCCTGGGCAACCGGCTC
3188 --+---------+---------+---------+---------+---------+------- 3247
     CTCACCAGGCGGCTCGCGCCGCCCGACAGGACGGACGACCGCCGGGACCCGTTGGCCGAG

     TGCGGGCCCGCCACGGCCGCCTGGGCGGGCAACTGGACCGGCGCCCCCGACGTCTCGGCG
3248 --+---------+---------+---------+---------+---------+------- 3307
     ACGCCCGGGCGGTGCCGGCGGACCCGCCCGTTGACCTGGCCGCGGGGGCTGCAGAGCCGC

     CTGGGCGCGCAGGGCGTGCTGCTGCTGTCCACGCGGGACCTGGCCTTCGCCGGCGCCGTG
3308 --+---------+---------+---------+---------+---------+------- 3367
     GACCCGCGCGTCCCGCACGACGACGACAGGTGCGCCCTGGACCGGAAGCGGCCGCGGCAC

     GAGTTCCTGGGGCTGCTGGCCGGCGCCTGCGACCGCCGCCTCATCGTCGTCAACGCCGTG
3368 --+---------+---------+---------+---------+---------+------- 3427
     CTCAAGGACCCCGACGACCGGCCGCGGACGCTGGCGGCGGAGTAGCAGCAGTTGCGGCAC

     CGCGCCGCGGACTGGCCCGCCGACGGGCCCGTGGTCTCGCGGCAGCACGCCTACCTGGCC
3428 --+---------+---------+---------+---------+---------+------- 3487
     GCGCGGCGCCTGACCGGGCGGCTGCCCGGGCACCAGAGCGCCGTCGTGCGGATGGACCGG

     TGCGAGGTGCTGCCCGCCGTGCAGTGCGCCGTGCGCTGGCCGGCGGCGCGGGACCTGCGC
3488 --+---------+---------+---------+---------+---------+------- 3547
     ACGCTCCACGACGGGCGGCACGTCACGCGGCACGCGACCGGCCGCCGCGCCCTGGACGCG

     CGCACCGTGCTGGCCTCCGGCCGCGTGTTCGGGCCGGGGGTCTTCGCGCGCGTGGAGGCC
3548 --+---------+---------+---------+---------+---------+------- 3607
     GCGTGGCACGACCGGAGGCCGGCGCACAAGCCCGGCCCCCAGAAGCGCGCGCACCTCCGG

     GCGCACGCGCGCCTGTACCCCGACGCGCCGCCGCTGCGCCTCTGCCGCGGGGCCAACGTG
3608 --+---------+---------+---------+---------+---------+------- 3667
     CGCGTGCGCGCGGACATGGGGCTGCGCGGCGGCGACGCGGAGACGGCGCCCCGGTTGCAC
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     CGGTACCGCGTGCGCACGCGCTTCGGCCCCGACACGCTGGTGCCCATGTCCCCGCGCGAG
3668 --+---------+---------+---------+---------+---------+------- 3727
     GCCATGGCGCACGCGTGCGCGAAGCCGGGGCTGTGCGACCACGGGTACAGGGGCGCGCTC

     TACCGCCGCGCCGTGCTCCCGGCGCTGGACGGCCGGGCCGCCGCCTCGGGCGCGGGCGAC
3728 --+---------+---------+---------+---------+---------+------- 3787
     ATGGCGGCGCGGCACGAGGGCCGCGACCTGCCGGCCCGGCGGCGGAGCCCGCGCCCGCTG

     GCCATGGCGCCCGGCGCGCCGGACTTCTGCGAGGACGAGGCGCACTCGCACCGCGCCTGC
3788 --+---------+---------+---------+---------+---------+------- 3847
     CGGTACCGCGGGCCGCGCGGCCTGAAGACGCTCCTGCTCCGCGTGAGCGTGGCGCGGACG

     GCGCGCTGGGGCCTGGGCGCGCCGCTGCGGCCCGTCTACGTGGCGCTGGGGCGCGACGCC
3848 --+---------+---------+---------+---------+---------+------- 3907
     CGCGCGACCCCGGACCCGCGCGGCGACGCCGGGCAGATGCACCGCGACCCCGCGCTGCGG

     GTGCGCGGCGGCCCGGCGGAGCTGCGCGGGCCGCGGCGGGAGTTCTGCGCGCGGGCGCTG
3908 --+---------+---------+---------+---------+---------+------- 3967
     CACGCGCCGCCGGGCCGCCTCGACGCGCCCGGCGCCGCCCTCAAGACGCGCGCCCGCGAC

     CTCGAGCCCGACGGCGACGCGCCCCCGCTGGTGCTGCGCGACGACGCGGACGCGGGCCCG
3968 --+---------+---------+---------+---------+---------+------- 4027
     GAGCTCGGGCTGCCGCTGCGCGGGGGCGACCACGACGCGCTGCTGCGCCTGCGCCCGGGC

     CCCCCGCAGATACGCTGGGCGTCGGCCGCGGGCCGCGCGGGGACGGTGCTGGCCGCGGCG
4028 --+---------+---------+---------+---------+---------+------- 4087
     GGGGGCGTCTATGCGACCCGCAGCCGGCGCCCGGCGCGCCCCTGCCACGACCGGCGCCGC

     GGCGGCGGCGTGGAGGTGGTGGGGACCGCCGCGGGGCTGGCCACGCCGCCGAGGCGCGAG
4088 --+---------+---------+---------+---------+---------+------- 4147
     CCGCCGCCGCACCTCCACCACCCCTGGCGGCGCCCCGACCGGTGCGGCGGCTCCGCGCTC

     CCCGTGGACATGGACGCGGAGCTGGAGGACGACGACGACGGACTGTTTGGGGAGTGA
4148 --+---------+---------+---------+---------+---------+---- 4204
     GGGCACCTGTACCTGCGCCTCGACCTCCTGCTGCTGCTGCCTGACAAACCCCTCACT
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INTERNATIONAL SEARCH REPORT

Interna l Application No
PCT/EP 94/04138

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/38 C12N15/86 C07K14/035 A61K39/245

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,93 15749 (SMITHKLINE BEECHAM BIOLOGICALS) 19 August 1993 see page 2, line 1 - line 34 see page 3, line 27 - line 37; claims 2,4-7,9 ---	1-3,7, 9-11
A	JOURNAL OF VIROLOGY, vol.61, no.4, April 1987 pages 1092 - 1097 COLTON A. SMIZH ET AL. 'Mutants defective in herpes simplex virus type 2 ICP4: Isolation and preliminary characterization' see page 1093, right column, paragraph 1 --- -/--	5,6

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

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'P' document published prior to the international filing date but later than the priority date claimed

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'X' document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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'&' document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 April 1995	02 -05- 1995

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Montero Lopez, B

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	VIROLOGY, vol.160, no.1, September 1987 pages 176 - 182 COLTON A. SMITH ET AL. 'Intertypic recombinants of herpes simplex virus types 1 and 2 infected-cell polypeptide 4' see abstract see page 176, left column, paragraph 2 - page 177, left column, paragraph 1 see page 178, left column, paragraph 3 - page 179, right column, paragraph 1	5,6,8
A	NUCLEIC ACIDS RESEARCH., vol.12, no.4, 21 February 1984, ARLINGTON, VIRGINIA US pages 2061 - 2079 J. LINDSAY WHITTON ET AL. 'Replication origins and a sequence involved in coordinate induction of the immediate-early gene family are conserved in an intergenic region of herpes simplex virus' see page 2062, paragraph 3 see page 2067, paragraph 2; figure 4 see page 2071, paragraph 3	5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 94/04138

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark : Although claim 11 is directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found muluple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Interna il Application No
PCT/EP 94/04138

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9315749	19-08-93	AU-B- 3495993	03-09-93